ABSTRACT

DARRYL CARSTENSEN: Cultivation-independent Identification of Phenol-degrading Bacteria in Activated Sludge by Stable Isotope Probing
(Under the direction of Michael Aitken)

The activated sludge process is the most commonly used process for municipal wastewater treatment in the United States. Molecular methods in microbiology have provided more detailed insight of the activated sludge process in recent years. However, most of these methods still rely upon culturing of isolates for characterization of the physiology of an organism and extrapolation of those characteristics to more complex systems for the study of environmental samples. Stable isotope probing (SIP) is a method which circumvents the requirement of culturing isolates to identify functionally relevant organisms in a system. SIP involves incubating a microbial community with a ^{13}C-labeled carbon source, then recovering ^{13}C-enriched nucleic acids by density-gradient ultracentrifugation.

In this study, SIP was applied to a phenol-degrading activated sludge community and followed by denaturant gradient gel electrophoresis (DGGE) fingerprinting and construction of 16S rRNA gene clone libraries for identification of phenol degraders by phylogenetic analysis. The effects on SIP results of pulse (spike) addition of phenol were compared to those of slow, continuous addition of phenol. Also, the effects of chemostat enrichment of the activated sludge community with phenol as a sole carbon source were examined. Sequences of major phenol-degrading bacteria closely related to two β-proteobacterial

sequences previously implicated in phenol-degrading activated sludge were recovered. However, the most abundant sequence associated with phenol degradation was related to organisms in the α-proteobacteria which have not been previously implicated in phenol degradation in waste treatment systems.

The method of [U-^{13}C]phenol addition in SIP incubations affected the degree of ^{13}C-labeling in the SIP experiment, with the spike-fed samples being more heavily labeled than the continuously fed samples. This finding can have implications for the design and interpretation of future SIP experiments.

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CHAPTER 1

INTRODUCTION AND BACKGROUND

Despite the centrality of the activated sludge process to wastewater treatment, relatively little is definitively known about the microbial ecology that governs it. Traditionally, wastewater treatment systems have been designed using aggregate parameters to characterize the waste streams – biochemical oxygen demand (BOD), total suspended solids (TSS), or total Kjeldahl nitrogen (TKN) for example. The microbial ecology of these systems has focused on operational changes, such as the establishment of aerobic and anaerobic zones, to select communities of organisms which perform such broadly defined biochemical operations as removal of BOD, TKN, or phosphorus. These aggregate parameters and biochemical operations provide practically useful information to the wastewater engineer and provide a basis for modeling of wastewater treatment systems, but more finely detailed information about the constituents of wastewater streams and the microbial interactions within biological treatment processes is needed in order for wastewater engineers to better design and operate those processes. Advances in molecular biology are providing an exciting new set of tools with which wastewater engineers can more finely characterize biological treatment processes.

We may obtain information about the composition of a microbial community *in situ* by interrogating the nucleic acids of the organisms composing the community. Nucleic acid-based methods are becoming increasingly common in the wastewater treatment field and are

providing a level of detail previously unavailable for biological treatment processes. A basic understanding of the chemical structure of nucleic acids and the central dogma of molecular biology is fundamental to the understanding of these methods. The central dogma states that genetic information stored in deoxyribonucleic acid (DNA) is transcribed to ribonucleic acid (RNA), which is then translated into protein. A deeper look into each step of this process will reveal the questions which we can answer from knowledge of the characteristics of an organism's nucleic acids.

The "blueprints" of an organism are contained in its DNA. If the genes encoding a protein necessary for a particular biochemical process are not available to an organism in its DNA, the organism cannot carry out that process. Therefore, an organism's genetic composition determines its phenotypic potential, and knowledge of its DNA can answer the question "what biochemical processes can this organism perform?" In wastewater engineering, particularly for industrial wastewater, the biochemical processes we are concerned with typically involve the metabolic transformation of a particular compound.

There are several types of RNA, two of which are most commonly targeted by these molecular methods. The type of RNA referred to in the central dogma is messenger RNA (mRNA). mRNA carries the instructions from DNA to ribosomes, the protein factories of cells, when a gene is expressed by the organism. With knowledge of an organism's mRNA we can partially answer the question "what biochemical process is the organism performing?" This question might only be partially answered because a protein may have more than one function and more than one protein could be responsible for a particular function. There is still a level of uncertainty about an organism's activity in the environment even with knowledge of its mRNA.

The other type of RNA of interest is ribosomal RNA (rRNA), which is one of the necessary components of a functioning ribosome. For several reasons, rRNA and the genes encoding for rRNA have been widely adopted as a phylogenetic marker (phylogeny refers to evolutionary relationship at the molecular level). All known organisms possess ribosomes and, consequently, rRNA. Because rRNA is ubiquitous there is little pressure for an organism to obtain the rRNA gene of another organism by horizontal gene transfer, so that a given rRNA molecule is likely to be unique to the organism possessing it. There are regions of the rRNA molecule and the rRNA gene which are highly conserved from one species to another, but there are also regions which vary greatly. The rRNA gene and rRNA remain constant enough (in the highly conserved areas) to be recognizable from one organism to another, but also differs enough that small evolutionary changes from one organism to another can be detected. Finally, rRNA and the rRNA gene contain enough information to make statistically significant comparisons between many rRNA molecules while providing a fine level of phylogenetic detail.

Two types of techniques may be used to study nucleic acids – hybridization techniques and amplification techniques. Both techniques rely on the ability of complementary nucleotide bases to form hydrogen bonds. Probes are oligonucleotides which bind, or hybridize, to specific, complementary sequences of bases in DNA or RNA. Several hybridization techniques which make use of labeled probes have been developed. Probes may be labeled with radioactivity, colored agents, or fluorescent agents. Amplification makes use of the polymerase chain reaction (PCR) to produce many copies of a piece of DNA in a test tube. In PCR, two short oligonucleotide primers are added in great excess to a sample of DNA. The nucleotide sequence of both ends of the target DNA segment must be

known in order to develop PCR primers. Primers for the rRNA gene may be designed to target a broad group of organisms or a more specific group of organisms, possibly a single species, by targeting highly conserved or highly variable regions of the rRNA gene, respectively. The primers bind to complementary regions of DNA and provide a starting point for extension of the DNA by the enzyme DNA polymerase. After 20 to 30 cycles of PCR, a 10^6- to 10^9-fold increase in the targeted segment of DNA is obtained. After amplification by PCR, the resulting product DNA can be subjected to a multitude of molecular assays.

Some microbial community analysis tools used in wastewater engineering to date have been fluorescent in-situ hybridization (FISH) and PCR followed by denaturant gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), or terminal restriction fragment length polymorphism (t-RFLP). FISH makes use of fluorescently labeled oligonucleotide probes to detect specific groups of organisms in intact microbial communities, thus showing spatial relationships between different organisms in microbial communities with the potential for enumerating those organisms in a sample. FISH requires *a priori* knowledge of an organism's gene sequence if a gene of that specific organism is to be investigated.

DGGE, TGGE, and t-RFLP are usually used in non-specific evaluations of microbial communities, unlike FISH, which is used to investigate specific organisms or genes within a community. DGGE (Muyzer et al., 1993) and TGGE (Watanabe et al., 1998) provide a community "fingerprint" (analogous to a chromatogram) or rough basis from which to compare the structure and estimate diversity of microbial communities. In DGGE or TGGE, a PCR product of a variable region of a gene flanked by conserved regions is amplified from

one conserved region to the next. This double-stranded PCR product is then subjected to gel electrophoresis in which a denaturant gradient is established either chemically (DGGE) or by increasing temperature (TGGE) across the gel. Ideally, DNA amplified from different organisms will denature (separate) at different locations along the gradient due to differences in attractive forces keeping the DNA double-stranded. When the DNA denatures it remains at one position in the gel and a band of DNA forms. The location and, to some degree, the intensity of different bands in the gel indicate the diversity and structure of the microbial community.

Another community fingerprinting technique is t-RFLP (Liu et al., 1997). In t-RFLP, one PCR primer is labeled with fluorescent dye. After the PCR is performed, the product is digested with restriction enzymes which cut the DNA at specific nucleotide sequences. The terminal restriction fragments (t-RF) can be distinguished from other restriction fragments by the detection of fluorescence, and the lengths of t-RFs can be determined by polyacrylamide gel electrophoresis. Amplified genes of differing sequence are likely to produce a t-RF of differing length. Consequently, an estimate of community diversity can be made and multiple communities can be compared for structural similarity with t-RFLP fingerprinting.

In addition to comparing microbial communities or identifying organisms within these communities, culture-independent molecular assays can be used to target functional genes. These functional genes are most commonly known from isolation and characterization of a pure culture to be responsible for a specific biochemical process. However, the environment of such isolates is often drastically different from and simpler than that which is encountered in complex systems such as soil or activated sludge. Consequently, isolation and characterization of a microorganism does not provide definitive

information of that microorganism's role in the more complex natural environment nor exclude the possibility that other functional genes are responsible for the same biochemical operation *in situ*.

Both cultured and uncultured microbes may be identified and enumerated using hybridization techniques such as FISH (Giovannoni et al., 1988; Moter and Göbel, 2000) or amplification techniques such as a quantitative polymerase chain reaction (qPCR) (Beller et al., 2002). But identification of these organisms in the environment neither provides a direct link between those organisms and a specific biochemical process such as metabolism of a target compound, nor does it exclude the possibility that uncultured and uncharacterized organisms may be responsible for the same process in the complex system. For example, Whiteley and Bailey (Whiteley and Bailey, 2000) used FISH to enumerate α-, β-, and γ-*Proteobacteria* and *Cytophaga-Flavobacterium* and used DGGE to obtain total community fingerprints within an industrial activated sludge system used to treat phenolic wastewater. While enumeration of these groups and an attempt to correlate changes in the bacterial community structure to changes in operating conditions within the treatment process is useful, no definitive link between the bacterial communities and phenol degradation can be made from such a study.

Watanabe et al. (Watanabe et al., 1998) explored the effects of isolation methods, including batch culture enrichment and chemostat enrichment of a municipal activated sludge which had been acclimated to phenol, on the selection of 16s rRNA genes and functional genes for the largest subunit of multicomponent phenol hydroxylases (LmPH) by using a combination of temperature gradient gel electrophoresis (TGGE) and sequencing of PCR products. Although an increase in prevalence of a specific gene in the community TGGE

fingerprint over the period of high selection pressure for phenol degraders might indicate that that gene belongs to a phenol-degrading organism, there is not direct evidence that the organism was degrading phenol in the activated sludge community. Additionally, other functional genes may be responsible for phenol degradation. This could have been a factor that contributed to the lack of amplification of an LmPH gene in 10 of 27 isolates.

A methodological approach to identify functionally relevant organisms from complex communities that circumvents the need to culture isolates is stable isotope probing (SIP). In SIP, a mixed culture is grown on a growth substrate labeled with a stable, non-radioactive isotope (such as ^{13}C) which occurs infrequently in natural samples. The microbes that utilize the "heavy" isotope-labeled substrate incorporate some of the heavy isotope into their cells, including macromolecular biomarkers such as DNA, RNA, and polar lipid fatty acids. Labeling of these molecules with the heavy isotope therefore provides a direct link between the structure (phylotype) and function (phenotype) of the microorganisms. The labeled macromolecules can be separated from unlabeled macromolecules by density gradient centrifugation (Radajewski et al., 2000) or identified without separation by secondary ion mass spectrometry (Orphan et al., 2001) or isotope ratio mass spectrometry (Boschker et al., 1998). The use of SIP, particularly DNA-SIP and RNA-SIP, has great potential to advance our knowledge of the microbial ecology of biological treatment processes (Manefield et al., 2004).

To date, the variables that can influence the outcomes of SIP experiments have not been fully explored. The primary objective of this study was to evaluate the effects of differences in incubation conditions on the identification of phenol-degrading bacteria by SIP. The underlying hypothesis was that pulse (spike) addition of phenol would select for

different organisms than would be selected by slow, continuous addition of phenol. A second objective was to evaluate the effect of chemostat enrichment on the selection of phenol-degrading bacteria in activated sludge from a municipal wastewater treatment plant.

CHAPTER 2

MATERIALS AND METHODS

Overview of Experiment

Activated sludge was collected from a municipal wastewater treatment plant operated by the Orange (County) Water and Sewer Authority (OWASA) in Chapel Hill, North Carolina. SIP with [U-^{13}C]phenol was performed with both a pulse (spike) addition and slow, continuous addition of the substrate with the activated sludge shortly after collection and with mixed liquor from a chemostat after 31 days of enrichment with phenol as the sole carbon source. An experimental overview is provided in Figure 1. DGGE was performed with the samples and clone libraries were made from selected samples in order to identify phenol-degrading organisms and monitor community changes after enrichment. Also, phenol degradation kinetic information was obtained for the enriched activated sludge to confirm the inhibitory nature of phenol and as a basis for estimating phenol concentrations during the SIP incubations. Chemostat operation, kinetic data acquisition, and SIP incubations were performed by undergraduate students at Elizabeth City (North Carolina) State University. Darryl Carstensen performed subsequent kinetic analysis, modeling of the SIP incubations, DGGE analysis, 16S rRNA gene clone library construction, and phylogenetic analysis of clones.

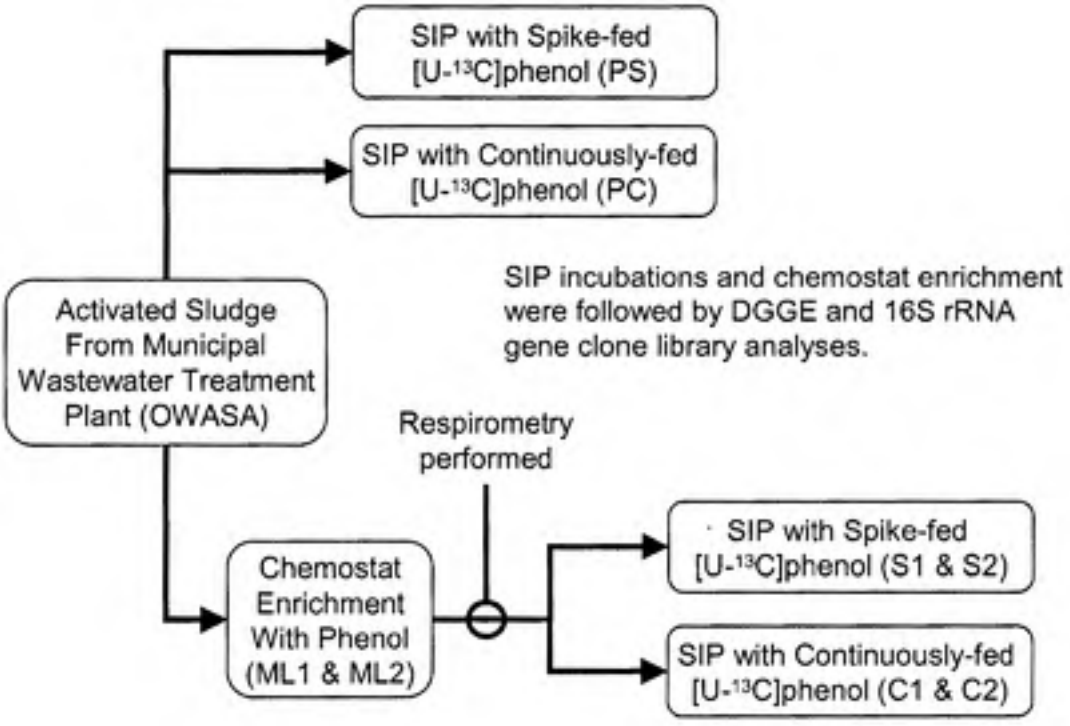

Figure 1. Experimental Overview.

Chemicals

[U-^{13}C]phenol (99%) was purchased from Cambridge Isotope Laboratories (Andover, Massachusetts) and stored in crystalline form. Other chemicals were reagent grade. Mineral salt media (MSM) consisted of 18.6 mM dibasic potassium phosphate (K_2HPO_4), 7.25 mM monobasic sodium phosphate (NaH_2PO_4), 37.4-mM ammonium chloride (NH_4Cl), 1.52 mM disodium nitrilotriacetic acid ($Na_2C_6H_7NO_6$), 0.81 mM magnesium sulfate ($MgSO_4$), 43 μM iron sulfate ($FeSO_4$), 18 μM manganese sulfate ($MnSO_4$), 10 μM zinc sulfate ($ZnSO_4$), and 4 μM cobalt chloride ($CoCl_2$).

TBE electrophoresis buffer consisted of 89 mM Tris base (Tris (hydroxymethyl)-aminomethane; $C_4H_{11}NO_3$), 89 mM boric acid (H_3BO_3), and 2 mM EDTA (ethylenediaminetetraacetic acid; $C_{10}H_{14}O_8N_2Na_2.2H_2O$) (1992).

Chemostat Enrichment

The chemostat was operated as a completely mixed flow reactor (CMFR) and inoculated with 300 mL activated sludge mixed in 300 mL sterile MSM. The chemostat consisted of a 1-L Spinner® flask (Bellco Biotechnology, Vineland, NJ) with magnetic stir-bar cap assembly into which a solution of phenol in MSM was pumped continuously at a rate of 360 mL/d. Mixed liquor (effluent) was also pumped continuously from the flask at the same rate as the feed solution to maintain a working volume of 600 mL, with a corresponding residence time of 1.7 days. The flask was mixed and aerated continuously. Influent phenol was loaded at a rate of 90 mg/d (0.360 L/d at a concentration of 250 mg/L). The chemical oxygen demand (COD) of phenol is 2.383 g oxygen/g phenol, so the COD loading rate was 214 mg/d. A schematic of the chemostat is shown in Figure 2.

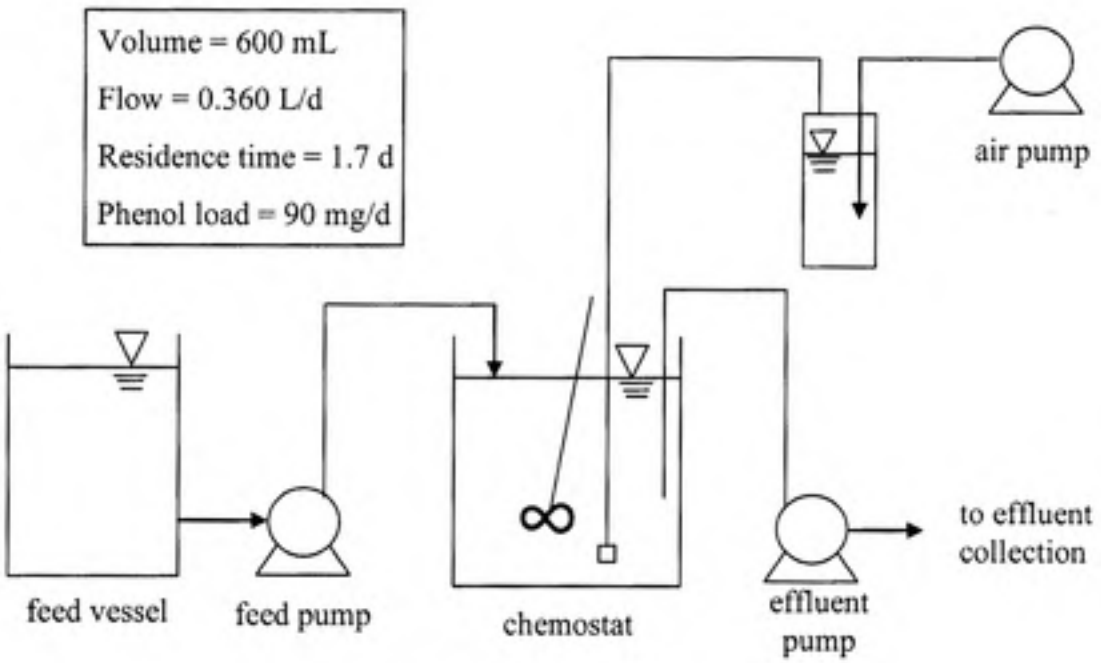

Figure 2. Schematic of Chemostat Operation.[MDA2]

Monitoring of Chemostat and Incubations

Total suspended solids (TSS) and phenol in the enrichment reactor effluent were measured in duplicate by Method 2540D and Method 5530D, respectively, of Standard Methods (1999) at several times throughout the course of chemostat operation. At these times the total and soluble chemical oxygen demand (COD) of the effluent were also measured by the Hach manganese III reactor digestion method without chloride removal (Hach Company, 2003)[MDA3]. The phenol remaining after SIP incubations was also measured.

Phenol Degradation Kinetics

Respirometry was performed on the chemostat culture after 29 days of chemostat operation by measuring the oxygen uptake rate (OUR) using the respirometry method described previously (Aitken and McCoy, 1993). This method involved measuring the initial rate of oxygen consumption in a small batch vessel containing a fresh sample of mixed liquor from the chemostat spiked with a concentrated solution of phenol. The OUR and TSS data were used to infer phenol uptake kinetic parameters for the inhibitory kinetics described by the Andrews equation:

$$q = \hat{q} \cdot \frac{S}{S + K_S + S^2/K_I} \tag{1}$$

where q is the specific substrate (phenol) removal rate, $\hat{q}$ is the maximum specific substrate utilization rate, S is the substrate concentration, K_S is the half-saturation constant, and K_I is the inhibition constant.

The observed yield on a COD basis, $Y_{obs,COD}$, was determined by dividing the average total effluent COD by the influent COD of phenol (effluent phenol was near zero). The observed yield on a TSS basis, $Y_{obs,TSS}$, was obtained by dividing the average effluent TSS by the influent phenol concentration.

The OUR measured by respirometry was used to calculate the phenol utilization rate (PUR) and q in the following manner:

$$OUR = 2.383 \cdot PUR \cdot \left(1 - Y_{obs,COD}\right) \tag{2}$$

$$q = \frac{PUR}{TSS} \tag{3}$$

where 2.383 g oxygen/g phenol is the chemical oxygen demand of phenol. The OUR is related to the amount of substrate oxidized for energy. When converting the OUR to a

substrate utilization rate (PUR in this case), the oxygen utilized will equal the portion of the substrate used for energy (i.e. not incorporated into biomass). Hence, the "1–*Y*" factor in equation 2. The TSS value used to determine q was the value measured closest to the time respirometry was performed.

The kinetic parameters were obtained by performing a 3-parameter ($\hat{q}$, K_S, and K_I) nonlinear regression using the program ProStat (Version 4.01b; Poly Software International, Inc.; Pearl River, New York).

The values of $\hat{q}$, K_S, and K_I were combined with the observed yield (TSS basis, $Y_{obs,TSS}$) and the TSS concentration to estimate phenol removal and growth simultaneously during the SIP incubation conducted after chemostat enrichment (post-enrichment SIP). The spike addition of phenol corresponds to a completely mixed, batch reaction, with the following mass balance equation for phenol:

$$\frac{dS}{dt} = -\hat{q}\left[X_o + Y_{obs,TSS}(S_0 - S)\right]\left(\frac{S}{K_S + S + S^2/K_I}\right) \tag{4}$$

where X_0 is the initial biomass concentration as TSS and S_0 is the initial phenol concentration. It is assumed in equation 3 that the increase in biomass concentration over the incubation period can be approximated by multiplying the net decrease in phenol concentration by $Y_{obs,TSS}$. For the continuous addition of phenol (completely mixed, semi-batch reaction), the mass balance equation is:

$$\frac{dS}{dt} = \frac{F_i(S_i - S)}{V_0 + F_i t} - \hat{q}\left[X_0 + Y_{obs,TSS}\left(\frac{F_i S_i t - S(V_0 + F_i t)}{V_0 + F_i t}\right)\right]\left(\frac{S}{K_S + S + S^2/K_I}\right) \tag{5}$$

where F_i is the flow rate and S_i is the phenol concentration in the added phenol solution.

Here, the net increase in biomass concentration is approximated by coupling the observed yield to the net mass of phenol removed and dividing by the reactor volume. The fourth-order Runge-Kutta method (Abell and Braselton, 2001)[MDA4] was used to solve equations 4 and 5.

Pre-enrichment SIP Incubation

Each pre-enrichment incubation was performed in a sterile 250-mL Erlenmeyer flask with 2 mL activated sludge as inoculum in 47 mL sterile MSM. [U-^{13}C]phenol in 1 mL sterile MSM was added as a 2.1-mg pulse to one incubation flask (SIP incubation "PS") while it was fed continuously via syringe pump at a rate of 2.5 mg phenol/d to another incubation flask (SIP incubation "PC") over a 20-hr period. Flasks were shaken at 40 rpm at room temperature throughout incubation. A pictorial overview of the SIP incubations is provided in Figure 3.

Post-enrichment SIP Incubation

Each post-enrichment incubation was performed in a sterile 250-mL Erlenmeyer flask with 25 mL chemostat mixed liquor as inoculum, 24 mL filtered chemostat effluent, and 1 mL [U-^{13}C]phenol stock solution at a concentration of 2.1 mg phenol/mL in sterile MSM. [U-^{13}C]phenol was added as a 2.1-mg spike to two incubation flasks (SIP incubations "S1" and "S2") while it was fed continuously at a rate of 2.5 mg phenol/d to another two incubation flasks (SIP incubations "C1" and "C2") over a 20-hr period. Flasks were shaken at 40-rpm at room temperature throughout incubation.

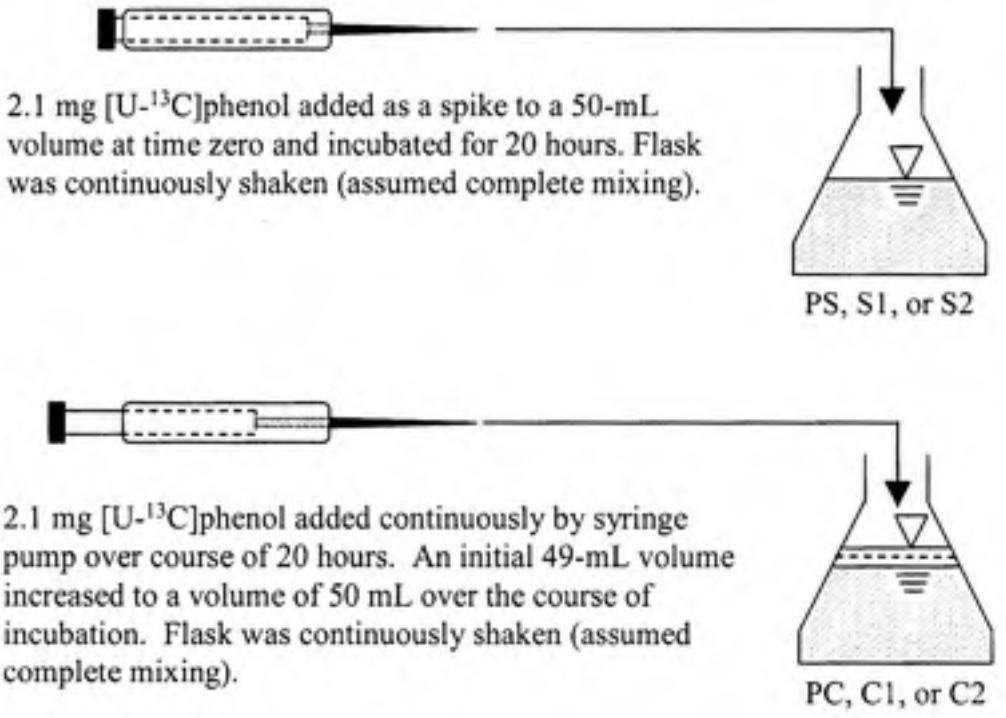

Figure 3. Overview of SIP Incubations

DNA Extraction

DNA extractions were performed with a MoBio (Carlsbad, California) UltraClean soil DNA extraction kit. After the pre-enrichment SIP incubation, 1 mL of the volume from each flask was syringe-filtered (0.22 μm) for analysis of residual phenol. The remaining volume was centrifuged at 10,000 rpm for 5 minutes and the pellet resuspended in 0.5 mL TBE buffer in a sterile microcentrifuge tube and frozen at -20°C for subsequent DNA extraction.

After the post-enrichment SIP incubation, 48 mL from each flask was pipetted into sterile 50-mL centrifuge tubes and centrifuged at 5,000 rpm for 10 minutes. A 1-mL aliquot of supernatant was retained from each tube for phenol quantification, and each pellet was

resuspended in 1 mL of supernatant in a sterile 2-mL microcentrifuge tube and frozen at -20°C for subsequent DNA extraction. A 48-mL volume of mixed liquor from the chemostat was treated similarly at this time (sample ML). The quality and quantity of recovered DNA was estimated by agarose gel electrophoresis.

Separation and Isolation of DNA

Separation of the unlabeled and ^{13}C-labeled DNA was achieved by density gradient ultracentrifugation in cesium chloride according to the method of Radajewski et al. (Radajewski et al., 2000), except that a Sorvall OTD60B ultracentrifuge with a TV-1665 vertical rotor and appropriate ultracentrifuge tubes were used. Separated DNA was recovered by collecting fractions from the bottom of each tube as described in Singleton et al. (Singleton et al., 2005). DNA recovered from SIP incubations PC, PS, C1, and C2 were separated into 400-µL fractions, while the DNA from SIP incubations C2 and S2 were separated into 100-µL fractions.

Molecular Analyses

PCR and construction of clone libraries for the 16S rRNA genes in DNA from the various samples was performed as previously described (Singleton et al., 2005) for the ML-, PC-, and S-clone libraries. The C-clone library was constructed using a TOPO TA Cloning® kit (Invitrogen; Carlsbad, CA). A total of 87 clones (40 ML-, 18 PC-, 23 S-, and 6 C-clones)[MDA5] were partially sequenced by SeqWright DNA Technology Services (Houston, Texas) using primer 8f. Close relatives of recovered sequences were determined by using the BLASTN program (Altschul et al., 1990) within the program suite of the

Genetics Computer Group (Wisconsin Package Version 10.3; Accelrys Inc.; San Diego, California). Sequences were grouped into a single operational taxonomic unit (OTU) with the program Sequencher (Version 4.5, Build 1415; Gene Codes Corporation; Ann Arbor, MI), using the dirty data algorithm, ReAligner gap placement optimization, 99% minimum similarity, and 20 base pair minimum overlap. The longest of the sequences within an OTU was selected to represent the OTU in phylogenetic analysis. Sequences were aligned using the pileup program of the Genetics Computer Group suite, and phylogenetic trees were constructed using the program ClustalX (Thompson et al., 1994). Trees were bootstrapped within ClustalX 1,000 times, and gaps were not considered during tree construction.

Sequences included on the phylogenetic tree in this study were submitted to GenBank and assigned accession numbers DQ322172 through DQ322206. These sequences are scheduled for release to the public database on December 19, 2005 pending processing by the National Center for Biotechnology Information (NCBI).

CHAPTER 3

RESULTS

Chemostat Operation

Activated sludge from a municipal wastewater treatment plant was used to seed a chemostat operating with phenol as the sole carbon source. TSS, total and soluble COD, and phenol in the effluent were monitored toward the end of chemostat operation. The results of these measurements are provided in Table 1 along with yields estimated from these measurements. Effluent phenol concentration was less than 0.40 mg/L in all measurements, corresponding to at least 99.6% removal across the chemostat. Effluent soluble COD was much higher than the effluent phenol COD, suggesting that there was substantial accumulation of soluble microbial products. Based on the mean TSS concentration and mass phenol removal rate, the specific rate of phenol removal was approximately 1.2 g phenol $(g\ TSS)^{-1}\ d^{-1}$. Based on the mean removal of dissolved COD and effluent TSS, the specific rate of COD removal was approximately 1.9 g COD $(g\ TSS)^{-1}\ d^{-1}$.

Table 1. Chemostat Measurements[a]

Parameter	Value[b]
Effluent Total COD (mg/L)	345 ± 44 (8)
Effluent Soluble COD (mg/L)	199 ± 55 (8)
Effluent TSS (mg/L)	128 ± 52 (8)
Effluent Phenol (mg/L)	0.23 ± 0.12 (7)
Effluent Particulate-COD to Effluent TSS ratio (g COD/g TSS)	1.2
Influent Phenol to Effluent TSS ratio (g phenol/g TSS)	2.0
$Y_{obs,COD}$ (g COD·(g phenol-COD)$^{-1}$)	0.58
$Y_{obs,TSS}$ (g TSS·(g phenol)$^{-1}$)	0.51

[a] Samples were collected on days 22, 27, 28, and 32.
[b] Values represent means and standard deviations. Number of replicates in parentheses.

Respirometry and Phenol Degradation Kinetics

After four weeks of chemostat operation, respirometry was performed (Figure 4) on the mixed liquor in order to estimate phenol degradation kinetic parameters. Figure 4 clearly illustrates that phenol exhibited substrate inhibition at concentrations near and above the effluent concentration from the chemostat. The results of the 3-parameter ($\hat{q}$, K_S, and K_I) nonlinear regression are provided in Table 2.

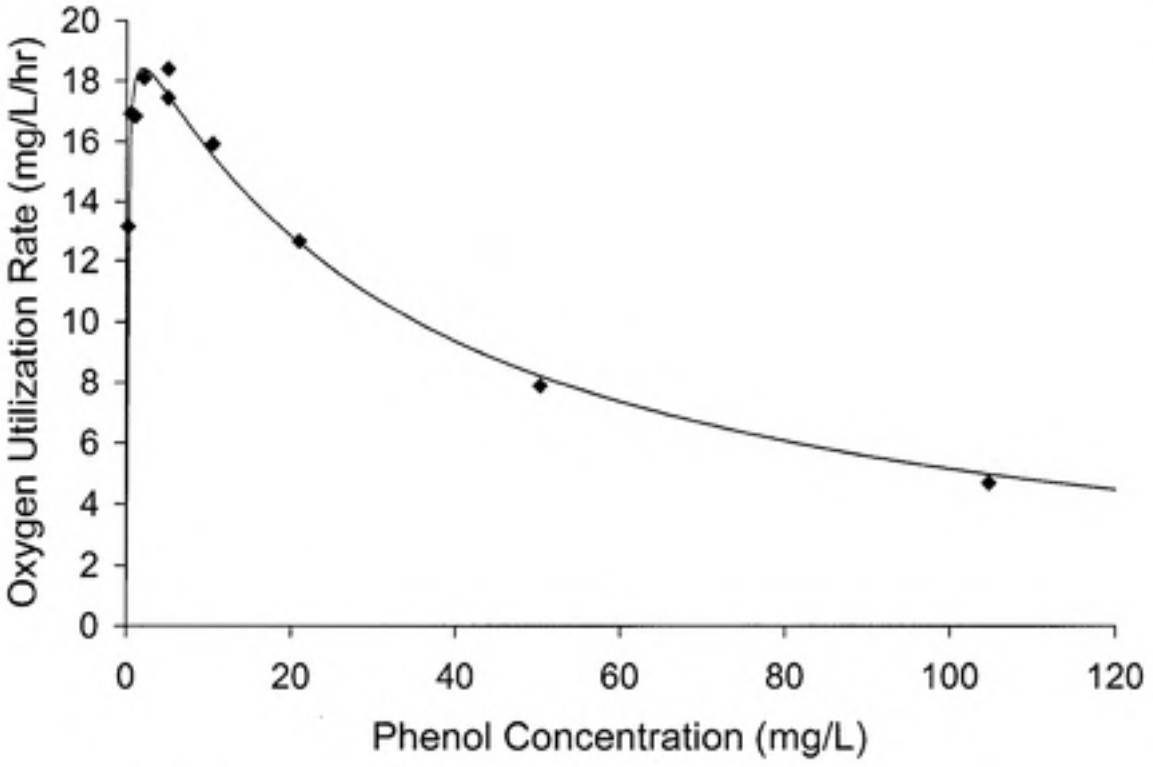

Figure 4. Mixed Liquor Respirometry Results. The lowest phenol concentration for which respirometry was performed was 0.21 mg/L, which was about the effluent concentration of phenol from the chemostat.

Table 2. Results of Nonlinear Regression of Respirometry Data.

Parameter	Value[a]
OUR_{max} ($mg \cdot L^{-1} \cdot hr^{-1}$)	20.6 ± 1.3
$\hat{q}$ (g phenol·(g TSS)$^{-1}$·hr^{-1})	0.111 ± 0.007[b]
K_S ($mg \cdot L^{-1}$)	0.12 ± 0.04
K_I ($mg \cdot L^{-1}$)	33 ± 8

[a]Fitted parameter values and 95%-confidence intervals.[MDA6]
[b]Value was calculated from the OUR_{max} value

SIP and DGGE of 16s rRNA Genes

Stable isotope probing (SIP) with [U-^{13}C]phenol was performed using activated sludge before chemostat enrichment and mixed liquor from the chemostat after 31 days of enrichment. In parallel incubations for each inoculum (activated sludge or chemostat mixed liquor), phenol was added either as a spike or continuously over a 20-hour period. Each method of phenol addition was evaluated in duplicate in the post-enrichment SIP experiment. Samples from the spike and continuous additions of phenol in the pre-enrichment SIP experiment were designated PS and PC, respectively. Samples from the post-enrichment experiment were designated C1 and C2 for the duplicate incubations with continuous phenol addition, and S1 and S2 for the duplicate incubations with the spike addition of phenol.

Modeling the post-enrichment incubations with the kinetic parameters obtained and equations 4 and 5 clearly shows that significant differences in phenol availability to the microbial communities resulted when the phenol was loaded either continuously or as a spike (Figure 5). A very low (near zero), steady-state concentration of phenol was calculated to exist throughout the course of the continuously fed incubations. Measurement of residual phenol concentrations at the end of the SIP incubation period confirmed that phenol concentrations were less than 0.6 mg/L or lower, corresponding to at least 98.7% removal of the added phenol, in all of the incubations.

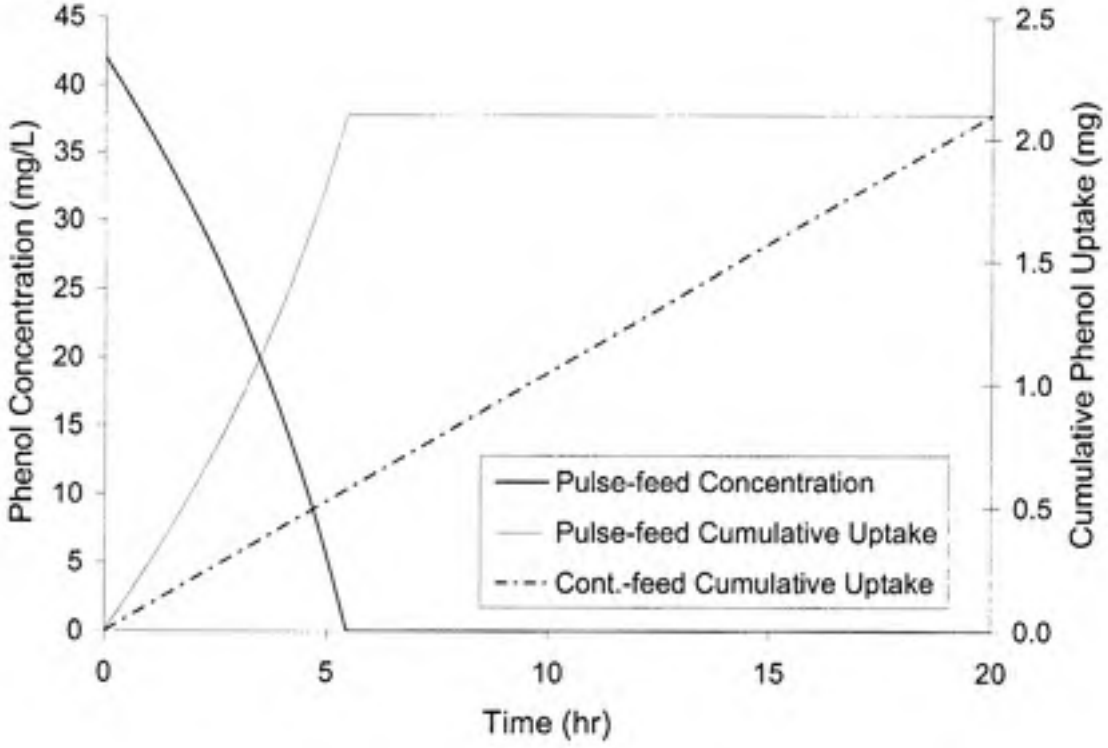

Figure 5. Post-enrichment SIP Kinetic Model Results.[MDA7] The lines represent the solutions obtained by numeric integration of equations 4 and 5. The phenol concentration remained near zero throughout the course of the continuously fed incubations (C1 and C2) and therefore was not plotted on this chart.

Samples PC, PS, C1, and S1 were separated into 400-µL fractions. A PCR was performed on fractions 4-8 of these samples (Figure 6) in order to visualize the separation of labeled and unlabeled DNA achieved during the SIP experiment. Fraction 4 was the densest and fraction 8 was the least dense fraction screened from each sample on the agarose gel shown in Figure 6. It is clear from Figure 6 that the manner in which the phenol was added, either as a spike or continuously, affected the degree of labeling achieved in the SIP experiment. Because no clear separation of the "heavy" and "light" DNA in the

continuously-fed samples was observed, the remaining samples – C2 and S2 – were separated into 100-μL fractions to evaluate whether separation between labeled and unlabeled DNA would be observable in sample C2 with finer fractionation. However, there was still no clear break between the heavy and light fractions of sample C2 (Figure A-2 in the Appendix).

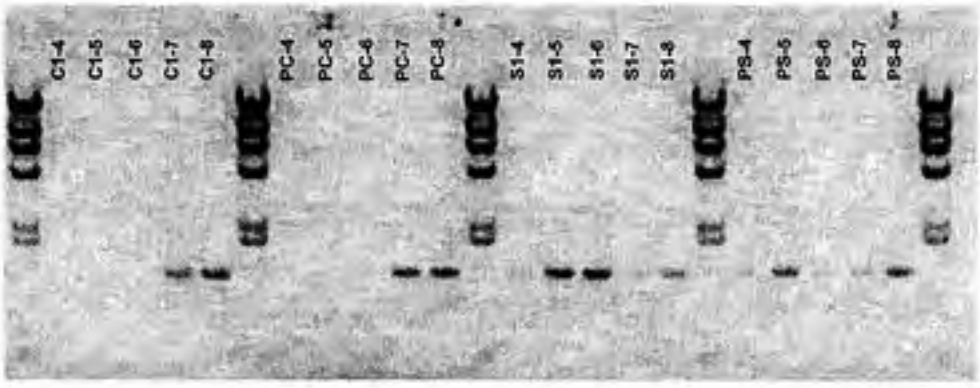

Figure 6. SIP Separation Results. The label at the top of each lane contains the SIP incubation identification followed by the fraction number. The lower fraction numbers correspond to lower positions in the ultracentrifuge tube and contain denser (more fully labeled) DNA than fractions of higher number from the same tube. This agarose gel of PCR products from SIP fractions of incubations PC, PS, C1, and S1 clearly shows that the spike-fed incubations led to greater incorporation of the ^{13}C into DNA (greater separation between the labeled and unlabeled DNA) than did the continuously-fed incubations, as manifested by the visible bands corresponding to lower fractions for the DNA from the spike-fed incubations.

The results of DGGE analysis of the various samples are shown in Figure 7.[MDA8] The post-enrichment chemostat mixed liquor (ML1 and ML2) profiles certainly appear to be more complex than the post-enrichment SIP heavy fractions (C1-7, S1-5, C2-11, and S2-7).

Although the bands from the pre-enrichment continuously fed SIP sample (PC) are faint, they do appear to indicate that differently structured communities existed before and after chemostat enrichment.

Although there was no clear separation of "heavy" and "light" DNA in the 100-μL fractions from sample C2, the DGGE profile of the lowest fraction containing DNA from sample C2 closely resembled the most heavily labeled fraction from S2 (compare fractions C2-11 and S2-7 in Figure 7) and showed less diversity than a fraction obtained from higher in the ultracentrifuge tube (fraction C2-15 in Figure 7). This suggests that some separation likely occurred in sample C2 and that the DNA in the lowest fractions from C2 containing observable DNA was relatively ^{13}C-enriched (in comparison to the fractions higher up the ultracentrifuge tube). A similar comparison could not be made for the pre-enrichment SIP samples because DNA from the spike-fed pre-enrichment SIP (sample PS) could not be amplified with the DGGE primer set. Consequently, fractions PC-7 and C1-7 are considered to be only nominally "heavy" fractions because of the unclear separation in fractionation of these samples.

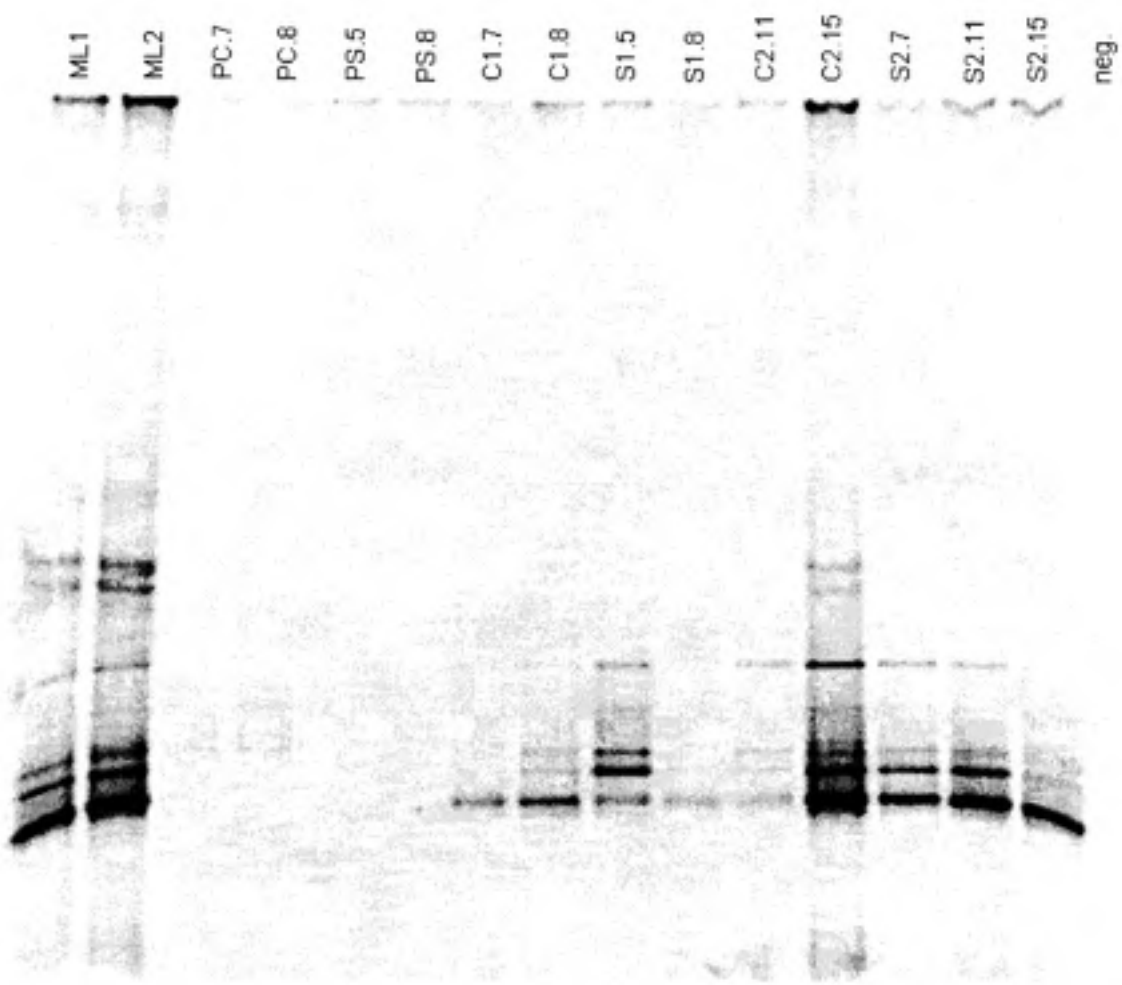

Figure 7. DGGE of SIP Fractions and Chemostat Mixed Liquor. The heaviest and lightest fractions which contained DNA from each sample and duplicates of the mixed liquor (ML1 and ML2) used to inoculate the post-enrichment SIP incubations (C1, C2, S1, and S2) are shown. The label at the top of each lane contains the incubation identification followed by the fraction number. The lower fraction numbers correspond to lower positions in the ultracentrifuge tube and contain denser (more fully labeled) DNA than fractions of higher number from the same tube.

Phylogenetic Analysis of 16s rRNA Gene Clone Libraries

16s rRNA gene clone libraries were constructed by randomly selecting 144 clones from SIP fractions PC-7, C2-12, and S2-7 and from sample ML2 (DNA extracted from the chemostat mixed liquor obtained at the same time as the post-enrichment SIP experiment) for sequencing. Useful sequence information was obtained from 40 clones from the sample of mixed liquor, 19 clones from fraction PC-7, 23 clones of fraction S2-7, and 6 clones from fraction C2-12. A clone library was constructed from fraction C2-12 rather than C2-11 after the volume of sample C2-11 was depleted from other analyses. Fraction C2-12 produced a very similar DGGE profile to fraction C2-11 (not shown) and was determined to be a suitable substitute for C2-11 for the heavy fraction in SIP incubation C2 on that basis.

A phylogenetic tree of the clone libraries, including the most closely related sequences obtained from a BLASTN search, is shown in Figure 8. The tree was rooted with the 16s rRNA gene from *Thermus aquaticus* (a thermophilic bacterium not likely to be closely related to bacteria found in activated sludge) as the outlier. The pre-enrichment clone library (PC) displayed greater phylogenetic diversity than both the mixed liquor after chemostat enrichment (clone library ML) and the heavy fractions of the post-enrichment spike-fed SIP incubations (clone libraries C and S). In turn, the mixed liquor had greater diversity than the heavy fractions from the post-enrichment SIP incubations, which is consistent with the DGGE analysis described above.

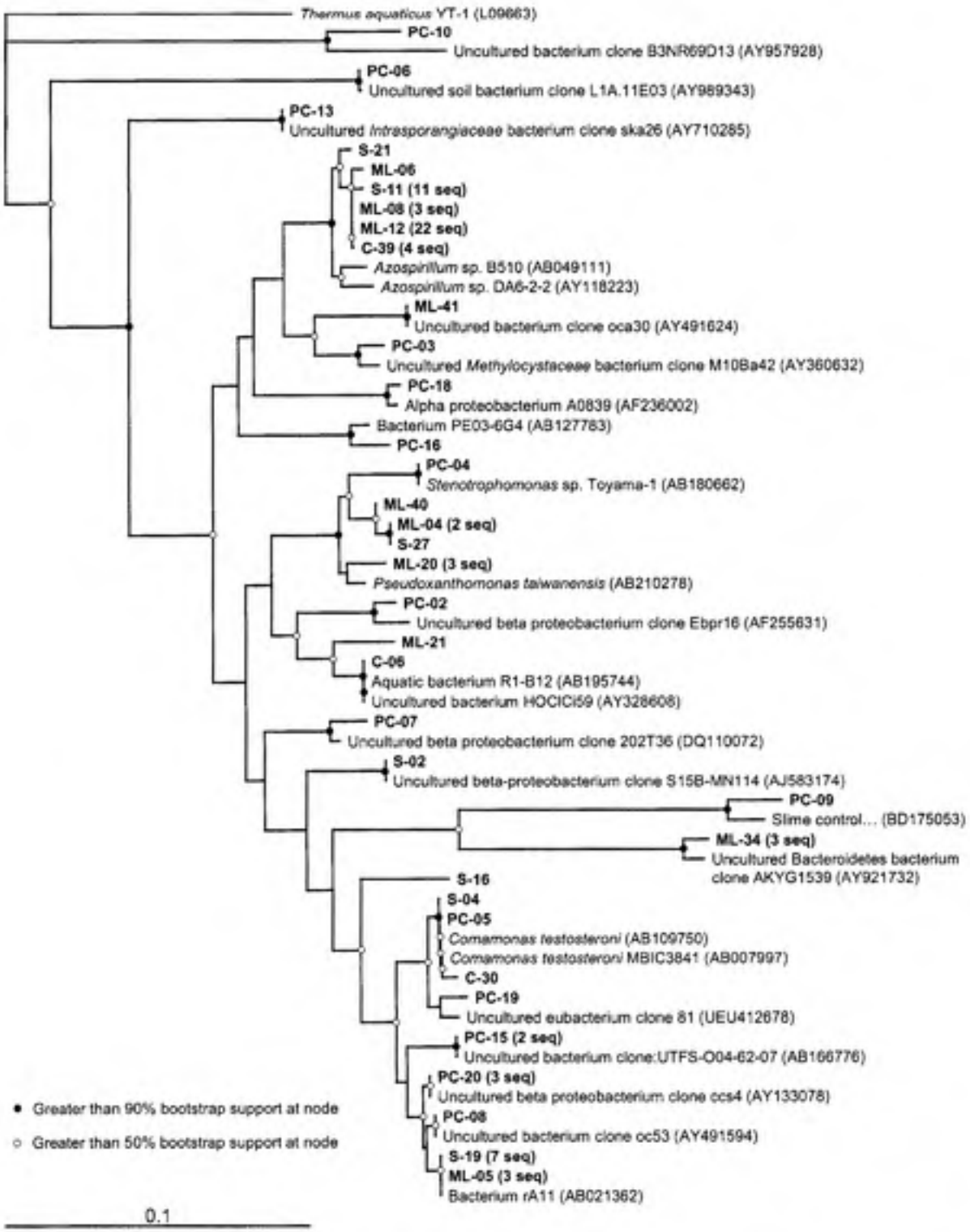

Figure 8. 16S rRNA Gene-based Phylogenetic Tree. The number of clones represented by a sequence designated as a single OTU is listed in parentheses after sequences which represent multiple clones. Sequences obtained by a BLASTN search are followed by their accession number in parentheses.

Table 3 shows the closest related sequence as determined by a BLASTN search to all sequences chosen to represent OTUs in this study. The sequences are grouped as determined by the Ribosomal Database Project II (RDP) (Cole et al., 2005) Classifier web-based program with the sequences obtained in this study (as opposed to those found in the BLASTN search) used as the input. Each clone from this experiment is listed under its most closely related sequence (or one of its most closely related sequences if multiple sequences were equally related). The clone identification is followed by the number of bases compared by BLASTN and the percent similarity in parentheses.

Of the 23 sequences obtained from the S clone library, one was highly similar to *Comamonas testosteroni* of the β-proteobacteria class and *Burkholderiales* order and was classified as 100% *Comamonas* by the RDP Classifier. Another OTU which represented 7 sequences was classified as 98% *Comamonas* by the RDP Classifier. Two OTUs were other types of *Burkholderiales* according to the RDP Classifier results, which is in general agreement with the phylogenetic tree grouping. Another of the S clone library OTUs was a γ-proteobacterium. Two of the OTUs were most similar to *Azospirillum* of the α-proteobacteria, one of which was the most abundant sequence type (11 of the 23 clones in the S clone library). In summary, accounting for OTUs with multiple sequences, 10 of 23 sequences were *Burkholderiales*, 12 of 23 sequences were similar to *Azospirilla*, and one was a γ-proteobacterium in the S clone library.

Table 3. Classification of 16S rRNA Gene Clones.

Most closely related sequence by BLASTN (Accession number)	Pre-enrichment SIP Continuous	Post-enrichment Chemostat Mixed Liquor	Post-enrichment SIP Continuous	Post-enrichment SIP Spike
α-Proteobacteria				
Azospirillum sp. DA6-2-2 (AY118223)		ML-06 (716; 94) ML-08 (444; 97) ML-12 (779; 97)	C-39 (580; 96)	S-11 (727; 97)
Azospirillum sp. B510 (AB049111)				S-21 (447; 96)
Uncultured bacterium clone oca30 (AY491624)		ML-41 (541; 100)		
Alpha proteobacterium A0839 (AF236002)	PC-18 (594; 98)			
Uncultured Methylocystaceae bacterium clone M10Ba42 (AY360632)	PC-03 (695; 97)			
Bacterium PE03-6G4 (AB127783)	PC-16 (424; 94)			
β-Proteobacteria				
Comamonas testosteroni MBIC3841 (AB007997)			C-30 (566; 94)	S-04 (844; 99)
Comamonas testosteroni (AB109750)	PC-05 (489; 98)			
Bacterium rA11 (AB021362)		ML-05 (808; 99)		S-16 (638; 98) S-19 (785; 99)
Uncultured eubacterium clone 81 (UEU412678)	PC-19 (721; 98)			
Uncultured bacterium clone oc53 (AY491594)	PC-08 (548; 99)			
Uncultured beta proteobacterium clone ccs4 (AY133078)	PC-20 (581; 99)			
Uncultured beta-proteobacterium clone S15B-MN114 (AJ583174)				S-02 (458; 100)
Uncultured bacterium clone:UTFS-O04-62-07 (AB166776)	PC-15 (592; 99)			

Table 3 (continued). Classification of 16S rRNA Gene Clones.

Most closely related sequence by BLASTN (Accession number)	Pre-enrichment SIP Continuous	Post-enrichment Chemostat Mixed Liquor	Post-enrichment SIP Continuous	Post-enrichment SIP Spike
β-Proteobacteria				
Uncultured beta proteobacterium clone 202T36 (DQ110072)	PC-07 (560; 94)			
Aquatic bacterium R1-B12 (AB195744)			C-06 (600; 99)	
Uncultured bacterium HOClCi59 (AY328608)		ML-21 (673; 96)		
Uncultured beta proteobacterium clone Ebpr16 (AF255631)	PC-02 (703; 97)			
γ-Proteobacteria				
Pseudoxanthomonas taiwanensis (AB210278)		ML-04 (807; 97) ML-40 (523; 93) ML-20 (653; 96)		S-27 (745; 97)
Stenotrophomonas sp. Toyama-1 (AB180662)	PC-04 (718; 99)			
Bacteroidetes				
Uncultured *Bacteroidetes* bacterium clone AKYG1539 (AY921732)		ML-34 (528; 95)		
Slime control method…neutral paper making (BD175053)	PC-09 (517; 95)			
Actinobacteria				
Uncultured *Intrasporangiaceae* bacterium clone ska26 (AY710285)	PC-13 (697; 99)			
Unclassified Bacteria				
Uncultured soil bacterium clone L1A.11E03 (AY989343)	PC-06 (571; 98)			
Uncultured bacterium clone B3NR69D13 (AY957928)	PC-10 (509; 90)			

The most numerically dominant OTU in the ML clone library, accounting for 26 of 40 sequences and most similar to *Azospirillum*, was also the dominant sequence in the S-library. The ML clone library had fewer sequences, 3 of 40 sequences, belonging to *Burkholderiales* (β-proteobacteria) and a higher number, 6 of 40 sequences, of γ-proteobacteria than the S clone library. Of the remaining sequences, one grouped with the α-proteobacteria not similar to *Azospirilla*, 3 grouped with *Bacteriodetes*, and one grouped with the β-proteobacteria.

Of the 6 sequences obtained from the C clone library, 4 were most similar to *Azospirilla*, one clustered with the *Burkholderiales*, and one grouped elsewhere within the β-proteobacteria.

Sequences of α-, β-, and γ-proteobacteria, *Bacteriodetes*, *Actinobacteria*, and unclassified bacteria were obtained from the PC clone library. No sequences closely related to *Azospirillum* were recovered, but one sequence of *Comamonas* and 7 other *Burkholderiales* sequences were found in the PC library.

CHAPTER 4

DISCUSSION

Based on clone library analysis of "heavy" DNA from SIP of an activated sludge community enriched on phenol, it appears that β-proteobacteria of the *Comamonas* genus and possibly another genus of the order *Burkholderiales* were important phenol degraders in this study. Sequences similar to *Comamonas* appeared to be the only sequences in common to DNA recovered from all "heavy" fractions from SIP incubation (PC, C, and S clone libraries), and therefore appear to be the only phenol-degrading bacteria in the microbial communities before and after chemostat enrichment. The importance of the β subclass of *Proteobacteria* for degradation of phenol in activated sludge has been suggested previously (Manefield et al., 2005; Watanabe and Hino, 1996; Watanabe et al., 1998). Bacterium rA11 (AB021362), a *Burkholderiales* sequence to which 8 sequences from the post-enrichment spike-fed SIP and 3 post-enrichment mixed liquor sequences were closely related, was isolated by direct plating from an activated sludge sample after 35 days of loading the activated sludge with 1.5 g phenol L^{-1} d^{-1} (Watanabe et al., 1999). The possible occurrence of phenol-degrading γ-proteobacteria (clone S-27, a singleton sequence) is also consistent with previous reports (Watanabe et al., 1998; Whiteley and Bailey, 2000).

It appears from this study that α-proteobacteria may potentially play a more important role in phenol degradation by activated sludge than has been found in previous studies (Cole et al., 2005; Watanabe et al., 1998; Whiteley and Bailey, 2000). The majority of sequences recovered from the chemostat mixed liquor (ML clone library), the post-enrichment continuously fed incubation (C clone library) and the post-enrichment spike-fed

incubation (S clone library) were most closely related to the α-proteobacterial genus *Azospirillum*. Strains of *Azospirillum* previously isolated from the rhizosphere and rhizoplane of different plants have displayed phenol-degrading capability (Barkovskii et al., 1995), but the potential importance of *Azospirillum* in phenol degradation by activated sludge has yet to be described.

Although it appears from the DGGE analysis that the community of phenol degraders enriched under the different feeding conditions in the post-enrichment SIP samples were similar (fractions C2-11 and S2-7 most clearly demonstrate this occurrence), the finding that the manner of ^{13}C-labeled substrate addition to a microbial community can affect the degree of labeling could have implications for further SIP studies. It is possible that a larger fraction of carbon is used for anabolism when the substrate is added as a spike because excess carbon and energy are available for some time after the spike, while a larger fraction is attributed to catabolism when the substrate is added slowly and continuously. When the substrate is added slowly and continuously, a low steady-state concentration of the substrate is maintained, potentially requiring that more of the carbon be used for maintenance of the cells rather than for growth. An alternative explanation for the reduced ^{13}C-enrichment of DNA during the incubation with continuous addition of phenol is that the slow availability of phenol permitted simultaneous utilization of exogenous, unlabeled carbon sources by the phenol-degrading bacteria. Concentrations of dissolved COD unrelated to phenol were high (Table 1), suggesting that there was a significant pool of unlabeled carbon potentially available for further assimilation. From the available data, it is not possible to distinguish between these alternative explanations for the reduced assimilation of ^{13}C during the SIP experiment with continuous addition of [U-^{13}C]phenol.

The method by which substrate is made available to a microbial community has been known for many years to influence the selection of organisms within the community. In waste treatment, classic studies illustrated that different communities are selected depending on whether the community is exposed to a substrate concentration gradient (Chudoba et al., 1991; Chudoba et al., 1985). This effect must require relatively long-term exposure to such conditions, however, since no qualitative differences in the phenol-degrading populations were observed between the spike-fed and continuously fed cultures over the 20-hour period of the post-enrichment SIP experiment.

In summary, β-proteobacteria of the genus *Comamonas* appear to be important phenol degraders as previously noted, but further research into the role of *Azospirillum* in phenol-degrading activated sludge is warranted. Also, incubation conditions must be carefully considered for their potential affects on microbial selection in future SIP experiments.

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APPENDIX

Table A-1. Chemostat Effluent Measurements.

	Parameter Value			
Parameter	Day 22	Day 27	Day 28	Day 32
Total COD (mg/L)	380; 340	411; 354	347; 262	318; 346
Soluble COD (mg/L)	271; 266	151; 145	147; 153	232; 225
TSS (mg/L)	50; 80	130; 180	190; 180	90; 120
Phenol Concentration (mg/L)	0.07; 0.12	0.25	0.20; 0.20	0.34; 0.40
pH	6.30[a]	6.35	6.36	6.03

[a]pH measurement taken on Day 20

Table A-2. Respirometry Results

Phenol Concentration, S_S ($mg \cdot L^{-1}$)	Oxygen Utilization Rate, OUR ($mg \cdot L^{-1} \cdot hr^{-1}$)	Specific Substrate Utilization Rate, q (hr^{-1})
0.21	13.2	0.071
0.50	16.9	0.091
1.05	16.9	0.091
2.09	18.1	0.098
5.03	17.5	0.094
5.03	18.4	0.099
10.5	15.9	0.086
20.9	12.7	0.068
50.3	7.89	0.043
105	4.70	0.025

Table A-3. Post-incubation Phenol Concentration of SIP Incubations

SIP Incubation	Phenol Concentration (mg/L)	Percent Removal
Pre-enrichment, Continuous (PC)	-0.20	100.5
Pre-enrichment, Spike (PS)	0.55	98.7
Post-enrichment, Continuous (C1)	0.45	98.9
Post-enrichment, Continuous (C2)	0.49	98.8
Post-enrichment, Spike (S1)	0.48	98.9
Post-enrichment, Spike (S2)	0.49	98.8

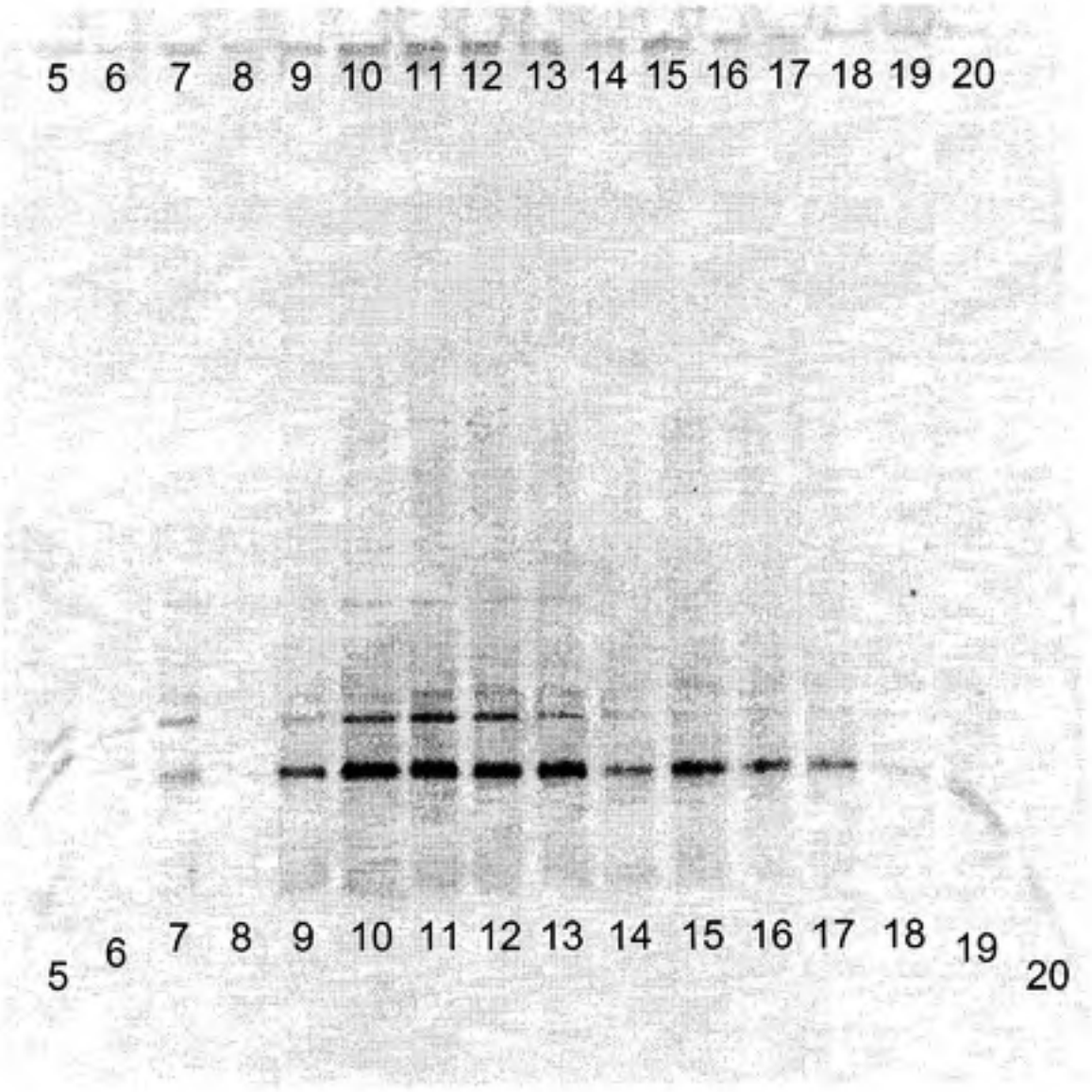

Figure A-1. DGGE of SIP Fractions from Incubation S2. Lower-numbered fractions contain more fully-labeled DNA than higher-numbered fractions.

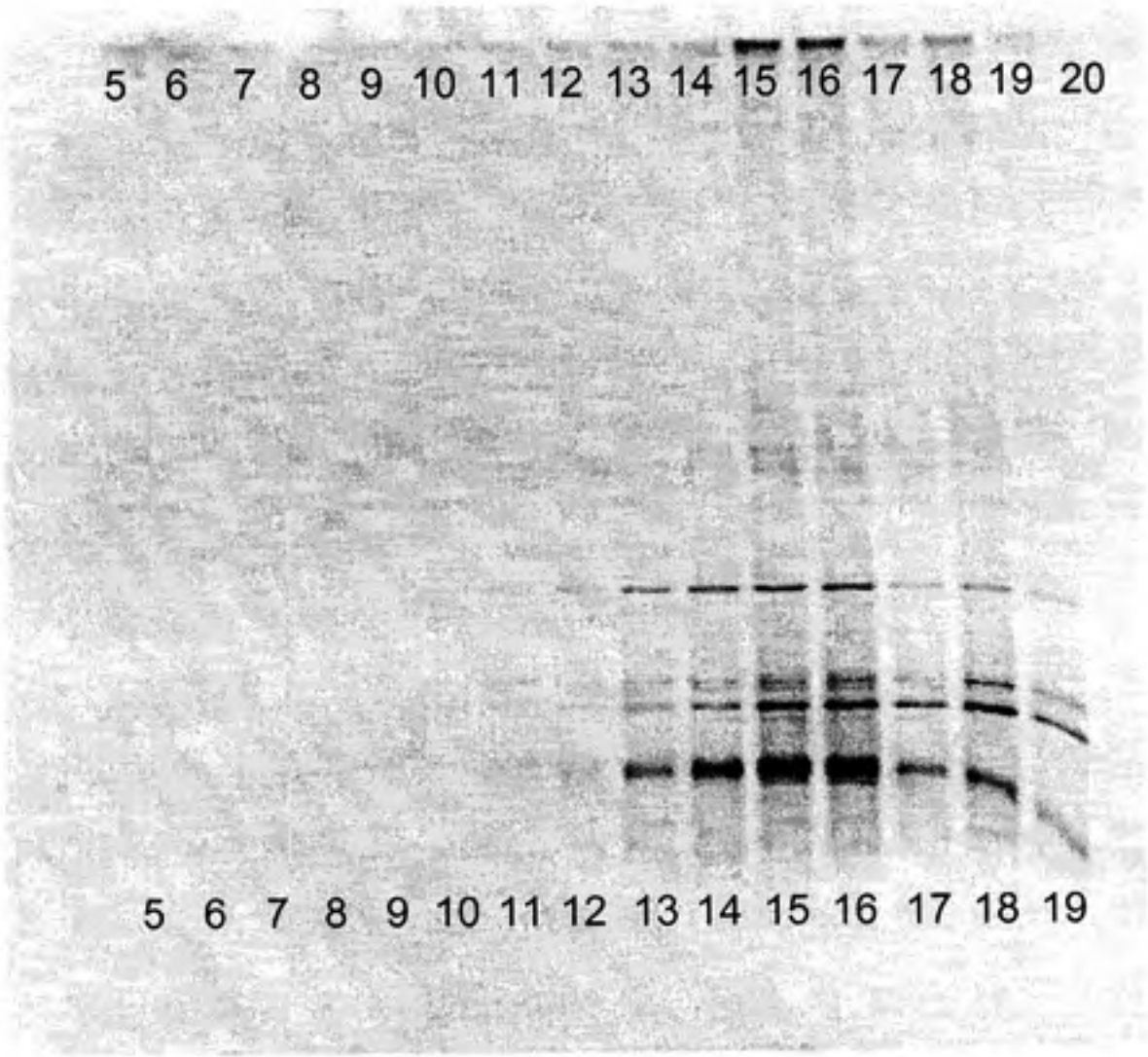

Figure A-2. DGGE of SIP Fractions from Incubation C2. Lower-numbered fractions contain more fully-labeled DNA than higher-numbered fractions.

Table A-4. Operational Taxonomic Unit (OTU) Groupings

Representative Clone	Number of Similar Clones	Similar Clones Comprising OTU
C-39	4	C-16, C-39, C-44, C-47
ML-12	22	ML-01, ML-03, ML-09, ML-10, ML-12, ML-13, ML-14, ML-16, ML-17, ML-19, ML-24, ML-25, ML-26, ML-27, ML-28, ML-29, ML-31, ML-32, ML-43, ML-44, ML-45, ML-46
ML-34	3	ML-33, ML-34, ML-36
ML-08	3	ML-02, ML-08, ML-35
ML-04	2	ML-04, ML-11
ML-05	3	ML-05, ML-07, ML-23
ML-20	3	ML-20, ML-37, ML-42
PC-20	3	PC-11, PC-20, PC-23
PC-15	2	PC-02
S-11	11	S-03, S-08, S-09, S-10, S-11, S-14, S-20, S-23, S-24, S-25, S-26
S-19	7	S-01, S-05, S-07, S-13, S-17, S-18, S-19

Table A-5. Accession Numbers of Sequences Obtained from Clone Libraries

Sequence ID (Accession Number)	Sequence ID (Accession Number)
C-06 (DQ322172)	PC-02 (DQ322185)
C-30 (DQ322173)	PC-03 (DQ322186)
C-39 (DQ322174)	PC-04 (DQ322187)
ML-04 (DQ322175)	PC-05 (DQ322188)
ML-05 (DQ322176)	PC-06 (DQ322189)
ML-06 (DQ322177)	PC-07 (DQ322190)
ML-08 (DQ322178)	PC-08 (DQ322191)
ML-12 (DQ322179)	PC-09 (DQ322192)
ML-20 (DQ322180)	PC-10 (DQ322193)
ML-21 (DQ322181)	PC-13 (DQ322194)
ML-34 (DQ322182)	PC-15 (DQ322195)
ML-40 (DQ322183)	PC-16 (DQ322196)
ML-41 (DQ322184)	PC-18 (DQ322197)
S-02 (DQ322200)	PC-19 (DQ322198)
S-04 (DQ322201)	PC-20 (DQ322199)
S-11 (DQ322202)	
S-16 (DQ322203)	
S-19 (DQ322204)	
S-21 (DQ322205)	
S-27 (DQ322206)	